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Application of Quantitative RT-PCR Using "TaqMan" Technology to Evaluate the Expression of CK 18 mRNA in Various Cell Lines

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Reverse transcriptase polymerase chain reaction (RT-PCR) is often used for sensitive detection of micrometastasis in peripheral blood, lymph nodes and bone marrow. While the utility of this method has been documented, it also has limitations in the detection of micrometastasis. The mRNA of target genes can be detected in healthy donors or in samples used for negative control, therefore the non-quantitativeness of conventional RT-PCR has been called into question.

We analyzed the expression level of cytokeratin (CK) 18 mRNA in established esophageal and gastrointestinal carcinoma cell lines and non-epithelial cells, using quantitative RT-PCR, based on real time 'TaqMan TM' technology. CK 18 mRNA is more highly expressed in carcinoma cells than in non-epithelial cells. However, the expression level in non-epithelial cells was easily detected using conventional RT-PCR and agarose gel electrophoresis. In an analysis of CK 18 mRNA expression in peripheral venous blood in 13 healthy volunteers, we found that CK 18 mRNA was much less expressed than in cancer cell lines. However, the expression in all samples was at a level which was also detected using conventional RT-PCR. It would thus seem that not only qualitative, but also quantitative analysis, of the target mRNA is important to detect micrometastasis. Quantitative RT-PCR methods will make comparisons of the possible differences in expression levels of the target gene. For clinical applications, much further study is needed.

Key Words: Quantitative RT-PCR, Real time PCR, TaqMan probe, Micrometastasis, Cytokeratin (CK) 18

Numerous reports have dealt with the detection of tumor cells circulating in peripheral blood and small numbers of cancer cells in bone marrow and in lymph nodes. Molecular biological approaches have facilitated the detection of minute numbers of cancer cells with a greater sensitivity. Immunohistochemical analysis using antibodies against antigens specifically expressed in epithelial or cancer cells is one method often used for this purpose. We reported that the micrometastasis of gastric cancer in bone marrow was detectable by immunostaining of cytokeratin (1, 2). However, it is difficult to detect circulating tumor cells in the peripheral blood using this approach. Reverse transcriptase polymerase chain reaction (RT-PCR) is widely used to detect the mRNA of various genes expressed in tumor cells. The sensitivity of micrometastasis detection by RT-PCR was found to be higher than when the immunohistochemical method was used (3, 4).

Common mRNA targets used for these purposes are CK 18, 19, 20, carcinoembryonic antigen (CEA), mucin-1 (MUC1) and others (3, 5-7). Cytokeratins are a subtype of intermediate filaments (IFs), predominantly expressed in epithelial tissues. Among the 20 types of human cytokeratins so far discovered (8-10), CK 18, CK 19 and CK 20 have been well studied. CEA is an oncofetal tumor antigen expressed in carcinoma cells, especially in gastrointestinal carcinomas.

Detection of micrometastasis using RT-PCR may be clinically important. However, extremely sensitive PCR methods can produce false positive results (11), and there are even reports on the limitations of this method in the detection of micrometastasis (12-15). The major reason for such limitations is that samples obtained from healthy donors show mRNA expression of such genes. This is partly attributed to the non-quantitativeness of the conventional RT-PCR method, in which PCR products are visualized by staining with ethidium bromide or autoradiograms, and for which

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Table I - Primers and probes sequences for quantitative RT-PCR of CK 18 and GAPDH

Target gene		Sequences 5'- -3'
CK 18	forward primer	ATCTTGGTGATGCCTTGGACA
	reverse primer	ACTTTGCCATCCACTATCCGG
	probe	(FAM)CATGCAAACCATCCAAAAGACCACCACC(TAMRA)
GAPDH	forward primer	GAAGGTGAAGGTCGGAGTC
	reverse primer	GAAGATGGTGATGGGATTTC
	probe	(FAM)CAAGCTTCCCGTTCTCAGCC(TAMRA)

detection depends on the number of amplification cycles and the design of the primers (11). If quantitative RT-PCR can be used for this type of analysis, it would be more reliable in the detection of micrometastasis because differences in intensity of mRNA expression can be detected.

Real time 'TaqMan TM' technology has been developed for highly quantitative PCR (16, 17). We analyzed the CK 18 mRNA expression in many established cell lines using this quantitative RT-PCR method. Then, we compared the expression levels among these cell lines. In addition, we first examined peripheral blood samples from 13 healthy volunteers and the application of this method for the detection of micrometastasis was given attention.

Materials and Methods

Cell lines and blood. DLD-1, LoVo, HCT 116, HT 29, SW 48, colorectal cancer cell lines, and MRC-5, WI 38, human normal fibroblast lines, were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). KATO III, MKN 45, MKN 74, NUGC-4, gastric cancer cell lines, were purchased from RIKEN Cell Bank (Tokyo, Japan). A primary culture of human aortic smooth muscle cells (HASMC), originally derived from an explant of a 22-year-old man, was purchased from Kurabo Industries Ltd. (Osaka, Japan). TE-1, TE-5, TE-10, TE-12, human esophageal cancer cell lines, were kindly provided by the staff at Tohoku University (Japan). MRC-5 and WI 38 cells were maintained in Minimum Eagle's Medium and HASMC cells were maintained in Dulbecco's Modified Eagle Medium, containing 1 mM of sodium pyruvate. HCT 116 and HT 29 cells were maintained in McCoy's medium. DLD-1, KATO III, MKN 45, MKN 74, NUGC-4, TE-1, TE-5, TE-10 and TE-12 cells were maintained in RPMI1640, and LoVo cells were kept in F12 medium. SW48 cells were maintained in Leibovitz's 15 medium. All the media used were purchased from Life Technologies Inc. (Rockville, MD, U.S.A.) and were supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, U.S.A.).

Ten milliliters of peripheral venous blood samples were taken from 13 healthy Japanese volunteers of both sexes who were 25-30 years of age.

Total RNA extraction and first strand cDNA synthesis. Total RNA was extracted from exponentially growing cells, using a commercially available RNA extraction kit (QIAGEN RNeasy Mini Kit for cell lines, QIAamp RNA Blood Mini Kit for blood samples; QIAGEN GmbH, Hilden, Germany) in accord with the recommendations of the manufacturer, but with some modifications. During extraction of RNA, treatment with DNase I (DNase I, FPLC pure, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) was added to avoid contamination of genomic DNA in the extracted RNA, which could affect the RT-PCR results. The quantity and quality of the extracted RNA was determined spectrophotometrically by absorbance at 260 nm and 280 nm. Synthesis of the first strand cDNA was done using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech Inc.) according to the manufacturer's protocol. Briefly, 2.0 μg of the total RNA was annealed with an oligo-d$(T)_{18}$ primer, and first strand cDNA was synthesized in a bulk first-strand cDNA reaction mixture, including Molony murine leukemia virus reverse transcriptase. The reaction was carried out at 37 °C in a water bath for 1 hr. The final volume of the first strand cDNA was 15 μl and 1 μl was used for each PCR reaction.

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Table II - Relative copies of CK 18 mRNA expressed in cell lines

Cell line		Relative copies of CK 18 mRNA mean ± S.D.
Cancer cells		
Colon cancer		
DLD-1	adenoca.	$6.47 \times 10^7 \pm 6.40 \times 10^6$
LoVo	adenoca.	$8.50 \times 10^7 \pm 5.43 \times 10^6$
HCT 116	adenoca.	$3.56 \times 10^7 \pm 1.07 \times 10^6$
HT 29	mod.dif.adenoca.	$7.87 \times 10^7 \pm 2.24 \times 10^6$
SW 48	adenoca.	$1.42 \times 10^7 \pm 1.06 \times 10^6$
Gastric cancer		
KATO III	signet ring cell ca.	$8.19 \times 10^7 \pm 5.27 \times 10^6$
MKN 45	poorly dif. adenoca.	$6.86 \times 10^7 \pm 2.31 \times 10^6$
MKN 74	mod.dif. adenoca.	$1.48 \times 10^8 \pm 3.46 \times 10^6$
NUGC 4	signet ring cell ca.	$4.56 \times 10^7 \pm 1.33 \times 10^6$
Esophageal cancer		
TE-1	well dif. s.c.c.	$9.31 \times 10^7 \pm 3.26 \times 10^6$
TE-5	poorly dif. s.c.c.	$1.75 \times 10^7 \pm 3.36 \times 10^5$
TE-10	well dif. s.c.c.	$7.66 \times 10^6 \pm 2.58 \times 10^5$
TE-12	mod. dif. s.c.c.	$9.37 \times 10^6 \pm 3.62 \times 10^5$
Non-epithelial cells		
Normal fibroblasts		
MRC-5		$2.40 \times 10^6 \pm 2.55 \times 10^4$
WI 38		$3.33 \times 10^6 \pm 1.93 \times 10^5$
HASMC		
HASMC586		$1.20 \times 10^6 \pm 6.17 \times 10^4$

Quantitative RT-PCR was done at least in triplicate for each sample.; The mean values of the relative copies and standard deviation of CK 18 mRNA are given.; S.D. : standard deviation; adenoca.: adenocarcinoma, dif.:differentiated, mod. : moderately, s.c.c. : squamous cell carcinoma.

Quantitative PCR using cancer cell cDNA. Quantification of mRNA levels of the target gene was made using real-time fluorescence detection 'TaqMan TM' technology and a Model 7700 Sequence Detector (Perkin-Elmer Corp., Foster City, CA, USA). Sequences of optimal primers and probes were determined using Software Primer Express provided by Perkin-Elmer Corp.

Fifty µl reactions contained: 300 nM of each primer, 200 nM specific TaqMan probe and 1 x TaqMan PCR Master Mix (Perkin-Elmer, Foster City, CA, USA), which contained Ampli-Taq DNA polymerase, a reaction buffer, dNTP, dUTP, and Amperase. The cycling parameters were: 2 min at 50°C, 10 min at 95°C followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. TaqMan GAPDH Control Reagent (Perkin-Elmer Corp.) was used for the RT-PCR of GAPDH mRNA, which served as an internal reference. The sequences of primers and hybridization probes for CK 18 and GAPDH are given in Table I. To compare the expression of CK 18 mRNA among cell lines, the ratio of relative copies of CK 18 mRNA to GAPDH mRNA was calculated. All analyses were made at least in triplicate.

In this quantitative PCR system, fluorescent emission is measured in real time and the calculated threshold values (Ct) reflect the starting target quantity, the lower Ct values of which reflect a greater number of starting target molecules (16). In this PCR reaction, a fluorescent-labelled oligonucleotide probe was added, in addition to the standard PCR components. The probe was complementary to the target sequence of interest and annealed during the PCR reaction. As Taq polymerase synthesizes the new strand, this probe

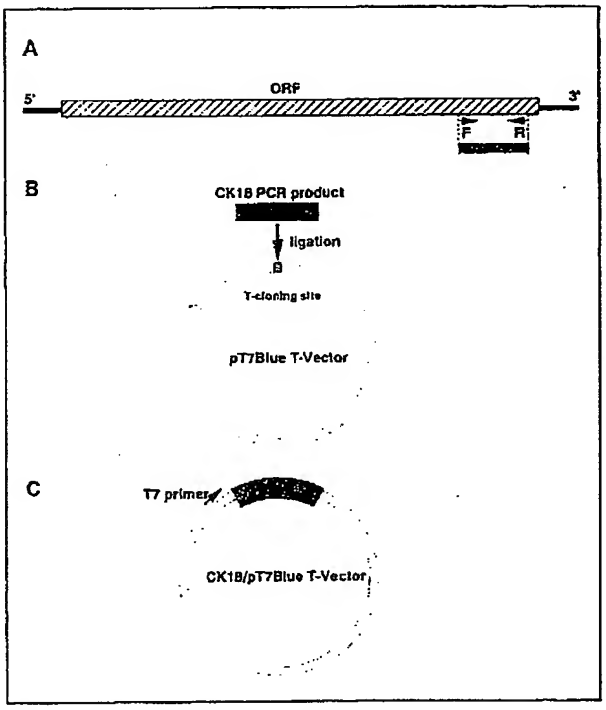

Fig. 1 - Schematic illustration of the construction of control plasmids for CK 18. (A) PCR was carried out using the same primers as were used for quantitative PCR to obtain the CK 18 fragment. F: forward primer, R: reverse primer (B) A CK 18 PCR fragment was ligated to a pT7Blue T-Vector. (C) The sequence of the ligated CK 18 fragment was checked using the T7 primer and an ABI 310 autosequencer.

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Table III - Relative copies of CK 18 mRNA expressed in the peripheral blood of healthy volunteers

No	Relative copies of CK 18 mRNA	Relative copies of GAPDH mRNA	CK 18 mRNA/ GAPDH mRNA
1	2.59×10^4	1.24×10^6	0.021
2	2.75×10^4	1.85×10^6	0.015
3	1.64×10^4	1.40×10^6	0.012
4	2.50×10^4	2.10×10^6	0.012
5	2.67×10^4	1.09×10^6	0.025
6	9.89×10^3	7.98×10^5	0.012
7	5.41×10^3	3.10×10^5	0.017
8	1.27×10^4	9.24×10^5	0.014
9	2.23×10^4	3.10×10^6	0.007
10	1.84×10^4	1.30×10^6	0.014
11	1.78×10^4	1.86×10^6	0.010
12	1.91×10^4	1.67×10^6	0.011
13	2.39×10^4	3.23×10^6	0.007

Quantitative RT-PCR was done at least in duplicate, for each sample.
The mean values of the relative copies of CK 18 mRNA and GAPDH mRNA are shown.
To compare differences in expression levels, the ratio of CK 18 mRNA/GAPDH mRNA was calculated.

is cleaved by its 5' to 3' nuclease activity and reporter fluorescence is emitted. Better specificity can be obtained due to the complementary nature of the probe, in addition to the primers. Relative gene expression was determined according to the standard curve (16, 17).

Quantitative RT-PCR using the cDNA of blood samples from healthy volunteers. Quantitative RT-PCR for CK 18 and GAPDH mRNA was done using cDNA synthesized using total RNA extracted from blood cells from healthy donors, using the same method as for cell lines.

Construction of plasmids containing a fragment of each target gene. To obtain a standard curve corresponding to CK 18 mRNA in quantitative real-time PCR, plasmids containing a fragment of CK 18 cDNA were constructed (Fig. 1). The PCR reaction was carried out to obtain the fragment, using a Perkin-Elmer GeneAmp PCR system 2400 (Norwalk, CT, USA). The first-strand cDNA of colon cancer cell lines was used as a template. The same primers, as those used in quantitative PCR, were used in this PCR reaction. PCR products were checked by agarose gel electrophoresis, and were ligated to a pT7Blue T-Vector (Novagen, Inc., Madison, WI, USA) with T4 DNA ligase (Takara, Tokyo, Japan). Ligation of the fragment into the plasmid was done using T-cloning methods (18). The cloned plasmid containing the PCR fragment was purified and the sequence of the ligated fragment was determined using an ABI Prism 310 Sequencer (PE Applied Biosystems, Foster city, CA, USA) to confirm the absence of mutations. A control plasmid for GAPDH mRNA was constructed using the same method.

Conventional PCR and agarose gel electrophoresis. Amplification of the CK 18 sequence using standard plasmids was done by conventional PCR. Each plasmid had one fragment of CK 18 cDNA. Identical primers for quantitative PCR were used. PCR reactions were performed using TAKARA Taq Reagent Kits (TAKARA Co. Ltd., Tokyo, Japan) and run in the Perkin-Elmer GeneAmp PCR system 2400. A 50 µl of reaction mixture contained 1 x reaction buffer, 350 µM of each dNTP, 15 pmol of each primer, 2.5 u of the

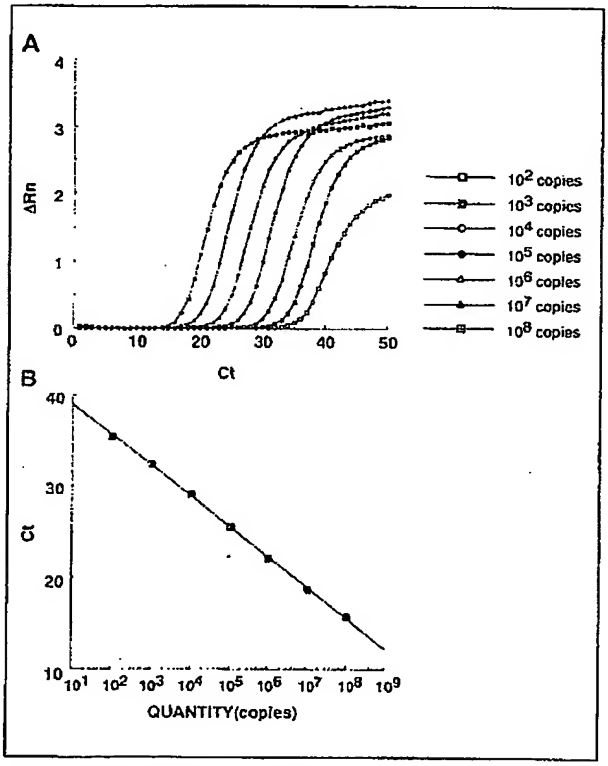

Fig. 2 - (A) Amplification plots for each sample containing known numbers of plasmid molecules carrying a CK 18 gene fragment.
(B) Number of plasmid molecules with the CK 18 gene fragment, plotted versus Ct.

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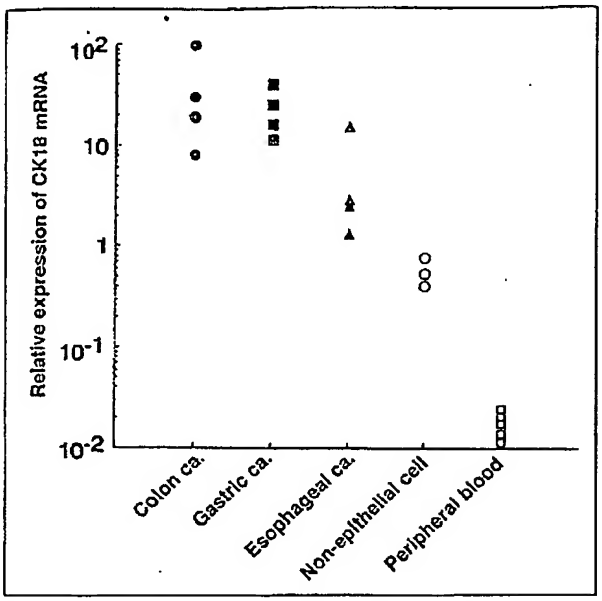

Fig. 3 - Relative expression of CK 18 mRNA in cancer cell lines, normal fibroblasts, human aortic smooth muscle cells, and peripheral venous blood cells of healthy volunteers. The ratio between relative copies of mRNA of each gene and GAPDH is shown.

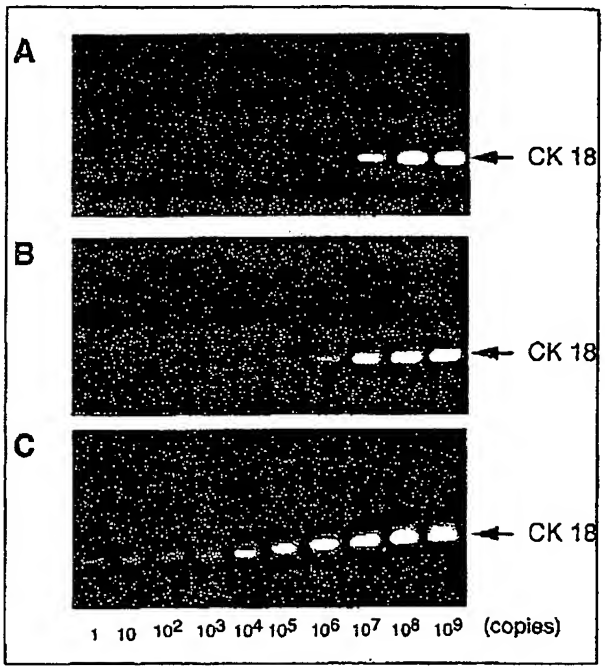

Fig. 4 - 1.8% agarose gel electrophoresis of CK 18 PCR using newly constructed control plasmids. The PCR thermal conditions were as described in Materials and Methods. The number of molecules (copies) included in one PCR reaction is given for each lane.
(A) 24 cycles, (B) 28 cycles, (C) 32 cycles

polymerase and various amounts of the control plasmid DNA. The thermal conditions of the system were as follows: one cycle at 95°C for 5 min; 24, 28 and 32 cycles at 95°C for 0.5 min, 60°C for 0.5 min, 72°C for 0.5 min; one cycle at 72°C for 10 min.

Fifteen µl of PCR product was analyzed using 1.8% agarose gel electrophoresis and visualized on a UV transilluminator, after staining with ethidium bromide (0.5 µg/ml).

Results

Standard curve of each target gene. We constructed control plasmids carrying sequences of CK 18 and GAPDH cDNA (Fig. 1), and a standard curve for each target gene was obtained using these plasmids. In Fig. 2A, amplification plots for each sample containing known numbers of plasmid molecules that carried a fragment of the CK 18 gene, are overlaid. The standard curve was constructed from plots for the calculated Ct values of each reaction containing a known number of plasmids (Fig. 1B). Fig. 1B shows that it is possible to quantify CK 18 mRNA expression in spans of six logs (from 10^2 to 10^8 molecules in each reaction). The same examination was made for GAPDH mRNA (data not shown).

Quantitation of mRNA expression of each target gene in cancer cell lines. Expression of CK 18 mRNA in various cell lines was then examined, using quantitative RT-PCR. The copy number was determined according to the standard curve obtained from the constructed plasmids (Table II). To compare the differences in expression of each gene, the ratio between relative copies of the mRNA of each gene and GAPDH was used (Fig. 3). As shown in Fig. 3, CK 18 mRNA expression levels differed little among colorectal and gastric cancer cells, while the level in the esophageal cancer cell lines was slightly lower than in those cell lines. The CK 18 mRNA expression level in normal fibroblasts and HASMC was lower than in cancer cell lines.

Expression of CK 18 mRNA in peripheral blood cells from healthy volunteers. We next analyzed the CK 18 mRNA expressed in peripheral venous blood cells from healthy volunteers (Table III). The ratio of CK 18 mRNA to GAPDH mRNA was calculated for comparison (Table III, Fig. 3). Compared with the CK 18 mR-

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NA level in cancer cell lines, normal fibroblasts or HASMC, the level detected in healthy blood samples was much lower (Fig. 3), although in all samples CK 18 mRNA was detected in the range of 5×10^3 - 2.75×10^4 in each reaction (Table III).

Conventional PCR and agarose gel electrophoresis. Thus, it was possible to quantitate the expression level of CK 18 mRNA in human cancer and non-epithelial cells and peripheral blood cells using 'TaqMan TM' technology. Next, we checked the results of conventional PCR, using the same control plasmids. Samples containing various numbers of control plasmids, 1 to 10^9, were amplified by PCR and identical primers used for the quantitative PCR were used. When PCR was done for 24 cycles, the PCR product for more than 10^6 plasmids could be visualized as a band. When the number of PCR cycles was increased, even the PCR product for only one copy could be recognized (Fig. 4C). Therefore, it is difficult to determine optical conditions and to quantify the amounts of PCR products if conventional PCR methods are used.

Discussion

We analyzed the CK 18 mRNA expression in esophageal and gastrointestinal cancer and non-epithelial cells, using quantitative RT-PCR using real time 'TaqMan TM' technology with a Model 7700 Sequence Detector (16). RT-PCR is now in wide use (6, 19-22) and cytokeratin (CK) 18, CK 19, CK 20, CEA have been well studied. These genes are thought to be expressed specifically in epithelial or carcinoma cells. However, there are reports of limitations with this method (11-15, 23), which show these genes are expressed even in blood samples from healthy humans or non-cancerous patients. Primer modifications (5, 24), or multiple step PCR (20), have been developed for specific and sensitive detection of micrometastasis. The extreme sensitivity raises the possibility that mRNA may be detected below levels where it has a physiological significance, or that the detection may be false positive. To minimize these problems, quantification of mRNA has been given attention. To achieve this, competitive PCR is now used. This method requires internal controls that are co-amplified with target genes. However, to quantify the amplified PCR products by comparison with internal controls, post-PCR manipulations, such as gel electrophoresis and visualization as bands for quantitation are needed. When more manipulations are added after PCR, the reliability for quantitativeness may well decrease. Considering these problems, quantitative RT-PCR using 'TaqMan' technology has advantages. No post-PCR manipulations are required with this method, and quantification and calculation of the results are all automated. In addition, without post-PCR manipulation, this method is simple and time-saving. These characteristics make it suitable for clinical application.

We described here the utility of quantitative RT-PCR for comparing the expression levels of target genes among different samples and detecting micrometastasis. We used only CK 18 in this study. To define the most specific and sensitive markers for detection of micrometastasis, a similar analysis for other genes, for example, CK 19, CK 20, CEA or other possible genes is necessary. There may be specific markers for organs in which cancer occurs, or specific for sites where micrometastasis will occur, such as blood, bone marrow or lymph nodes. Moreover, to apply this quantitative analysis for detection of micrometastasis clinically, it will be necessary to determine the cut-off value for each target gene. Further analysis, including larger numbers of healthy human subjects, as well as cancerous and non-cancerous individuals is in progress.

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